Chemically-Modified Inulin as a Polymeric Vaccine Carrier

UNDERGRADUATE HONORS THESIS

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Abstract

The use of polymers to enhance the delivery of vaccines is a growing area of research. Microparticles formed from polysaccharides can be formulated into vaccines by encapsulating a protein target (an antigen) and an immune danger signal (an adjuvant). The adjuvant is needed since most synthesized or subunit vaccines lack the immunologic potency that the entire pathogen would stimulate. Immunostimulatory polymers such as Inulin obtained from *dahlia* root could be used in place of a separate adjuvant. Inulin can be used as a base material to form chemically-modified polymers, such as acetalated (Ace) or trimethylsilyl (TMS) Inulin. To this end, the degradation of chemically modified Inulin microparticles of varying reaction times and modifications was tested at pH 5 and 7.4 using a spectrophotometer. The results showed a much slower release in extracellular conditions (pH 7.4) with a burst release at pH 5, corresponding to the acidity of the phagosome of phagocytic cells, which are the gatekeepers to a vaccine-mediated immune response in vivo. Additionally, the immunostimulatory effect of Inulin microparticles was evaluated using a TNF-α ELISA and the toxicity was evaluated using an MTT assay. Macrophages were incubated with five different concentrations (1000, 500, 250, 100, 50 μg/ml) of various types of Inulin microparticles (20 min Ace-Inulin, 24 hr Ace-Inulin, TMS-Inulin). Results show that the inulin microparticles are biocompatible and display a dose-dependent increase in TNF-α production. Currently, groups of inulin microparticles loaded with ova-albumin (a sample antigen) are being tested in vivo. Blood samples are being withdrawn from mice every 2 weeks, with a final sample at the end of six weeks. These samples will be tested using antibody titers, flow cytometry, and antigen recall.

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Introduction

Vaccination aims to generate a strong immune response against a selected antigen to provide long-lasting protection against infection.[1] Subunit vaccines (e.g. protein-based) offer numerous advantages over attenuated and heat-inactivated vaccines. Attenuated (live) viruses cannot be broadly applied due to safety risk associated with many diseases. Heat inactivated vaccines are safer but lack efficacy in generating a strong immune response. Subunit vaccines are considered to be a safer alternative to live or attenuated vaccine formulations because they can establish protective immunity, yet lack the molecular machinery to cause an infection.[2]

Currently, aluminum salts are the most commonly used vaccine adjuvants in FDA-approved vaccines. However, alum salts have been shown to induce allergenicity[3], form granulomas at injection sites[4], and only stimulate humoral responses[5,6]. The optimal vaccine should activate both the humoral and cellular arms of adaptive immunity.[7] Additionally, alum-adjuvanted vaccines have been shown to lose potency upon lyophilization, which could create an issue with long-term storage of the formulation.[8]

An increasing amount of research has investigated the use of polymers to improve delivery of vaccines. Microparticles fabricated from FDA-approved polymeric biomaterials including α-hydroxy acids such as poly(lactic-co-glycolic) acid (PLGA) are used broadly. However, PLGA has a slow degradation rate that is not ideal for delivery of antigenic payloads.[9] Additionally, the pH inside PLGA microparticles is acidic and provides an uncontrolled pH environment which can be an issue for antigen stability.[10] For use in drug delivery and controlled release applications, polymers sensitive to external stimuli such as magnetic fields, temperature, electric field and pH have been developed. An important channel to achieve controlled release

involves using pH-sensitive polymers to fabricate microparticles and nanoparticles that capitalize on natural pH differences *in vivo*.[11] pH-sensitive polymers have been used to target tumors[12,13], facilitate escape from endolysosomal pathways[14], and for uptake in the GI tract[15]. A pH-sensitive carrier allows for protein to be expediently delivered to antigen presentation systems due to the pH gradient in the endocytic pathway.[16, 17]

Acetalated polymers have been shown to have pH-sensitive characteristics ideal for delivery of vaccine elements to immune cells.[18] The degradation rate of acetalated dextran is easily tunable through the reaction time used to synthesize the polymer.[19] Additionally, silylated polymers are a recently developed biodegradable polymer that have many of the same features as acetalated polymers.[20] These polymers can be easily formed into microparticles using standard emulsion techniques since they become soluble in organic solvents. Microparticles produced from polysaccharides can form vaccines by encapsulating a protein target (an antigen) and an immune danger signal (an adjuvant). The adjuvant is often needed since most synthesized or subunit vaccines lack the immunologic potency that the entire pathogen would have stimulated.[21] Immunostimulatory polymers such as inulin obtained from dahlia root can act as the antigen delivery vehicle and could be used in place of including an additional adjuvant drug.

To improve long-term storage of vaccine formulations, plant polysaccharides such as inulin can be used to prevent the degradation of proteins during lyophilization and storage.[22] Inulin is a biocompatible polymer used in the food industry[23] and has been used clinically to measure renal function.[24] Specific isoforms of inulin have been shown to activate the alternative complement pathway.[25] Inulin-based adjuvants have successfully been tested in multiple animal models in combination with such antigens such as diphtheria, tetanus toxoid and hepatitis B.[1] Inulin-derived adjuvants have also been shown to promote both Th1 and Th2 immune responses

in vivo.[26] Inulin is an optimal adjuvant due to its biocompatibility, ability to promote both a cellular and humoral immune responses, and its cryoprotectant properties.

This study aims to use an immunostimulatory polymer (inulin) to produce a more effective vaccine carrier that does not require the addition of a separate adjuvant. Inulin has been shown to be biocompatible and is commonly used in food and clinical settings. Chemical modifications of the inulin allow for easy fabrication of delivery vehicles with pH-sensitive properties optimal for vaccine delivery. Inulin also serves as a cryoprotectant and prevents degradation of protein.[22] Additionally, inulin microparticles deliver antigens in particulate form which has been shown to be more immunogenic than the soluble form and to result in increased antigen uptake.[27,28]

Materials and Methods

Materials.

All materials were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise noted.

Acetalated Polymer Synthesis

Ace-Inulin (Ace-IN) was reacted in a similar method as used by Kauffman et al.[29] Briefly, lyophilized inulin (0.936 g, MW = 5,000) and pyridinium p-toluenesulfonate (0.0617 mmol) were dissolved in anhydrous dimethyl sulfoxide (DMSO, 10 mL) and then reacted with 2-ethoxypropene (37 mmol, Waterstone, Carmel, IN) under nitrogen gas. For longer reaction times, chloroform (4 mL) was added to the reaction mixture after one hour. The reaction was quenched with triethylamine (TEA, 1 mL) at set timepoints (20 min, 24 hr, 72 hr). The product was then precipitated in basic water (0.02% v/v triethylamine in water, pH 9), isolated using vacuum filtration, and lyophilized. The polymer was further purified by dissolving in ethanol (200 proof) and centrifuging (5 min, 10,000 × *g*, Thermo Legend Micro 21). The supernatant was precipitated again in basic water, filtered, and lyophilized to yield Ace-IN polymer (1 g), a white powder.

TMS-IN Polymer Synthesis

Inulin (0.5 g) was dissolved in anhydrous DMSO (5 mL). Triethylamine (1.2 g, 23.6 mmol / gram inulin) was added dropwise. The silylation of the inulin was performed by dropwise addition of the TMS-Cl (1.2 g, 22.4 mmol / gram Inulin). The reaction mixture was stirred at room temperature under nitrogen gas. After 30 min of reaction, 5 mL anhydrous chloroform was added, and the reaction was allowed to proceed for two more hours. Following the 2.5 hr, the reaction was quenched by dilution with 200 mL chloroform. The organic layer

was washed with saturated sodium bicarbonate solution followed by deionized water, dried over anhydrous sodium sulfate, and TMS-IN was collected as a white solid after lyophilization.

Particle Synthesis

Ace-IN OVA-loaded microparticles were prepared using a double-emulsion technique (water/oil/water) as previously described with Ace-DEX.[10] Briefly, a stock solution of ovalbumin (OVA) in PBS (200 μL) was prepared. 100 mg of Ace-IN was dissolved in dichloromethane (DCM, 1 mL) and added to the OVA solution. This two-phase mixture was then homogenized for 30s (IKA-25 Ultra Turrax Homogenizer, S25N-18G head, 18,000 RPM). 12 mL of 3% poly (vinyl alcohol) (PVA) in PBS (12 mL) solution was then added to the emulsion formed and homogenized using the same settings. The resulting double emulsion was immediately poured into a spinning solution of 0.3% PVA in PBS (40 mL). After two hours of spinning to evaporate the solvent and allow for particle hardening, the mixture was centrifuged (10 min, 14,000 × g). The supernatant was discarded, and the resulting sediment was washed with basic water (pH 9) and centrifuged under the same conditions several times in order to remove excess PVA. Finally, the microparticles were suspended in basic water (pH 9) and lyophilized for two days to yield Ace-IN microparticles, a white powder.

Acetalated Dextran (Ace-DEX) microparticles were prepared with the same procedure as described above, except Ace-DEX was used in place of Ace-IN. This Ace-DEX polymer (MW = 71k) was synthesized as previously described in Kauffman et al.[29]TMS-IN microparticles were also prepared with the same procedure, except TMS-IN polymer was used in place of Ace-DEX. Empty Ace-IN and TMS-IN Particles that did not contain protein were made in the same manner as the double-emulsion particles omitting the first emulsion with the OVA solution.

Particle Degradation

Empty Ace-IN and TMS-IN particles were suspended in triplicate at a concentration of 3 mg/mL in either a sodium acetate buffer (pH 5.0) or PBS (pH 7.4) and incubated at 37 °C on a shaker plate at 200 RPM. At various timepoints (0 to 336 hours) samples were analyzed using a spectrophotometer (600 nm). A standard curve was created with a range in concentrations of Inulin microparticles in PBS to determine percent degradation of the particles.

Quantification of Encapsulated OVA

Blank and OVA-loaded particles were prepared at 3 mg/mL in 1% (w/v) Sodium Dodecyl Sulfate (SDS) in 0.3 M sodium acetate buffer (pH 5.0). The solutions were incubated at 90 C until complete degradation of the particles was observed. A standard curve using free OVA was treated the same way. 150 uL of the samples/standards were reacted with 50 uL of 3 mg fluorescamine / mL in DMSO for 15 min. The fluorescence was read at Ex:390 / Em:470 nm.

Scanning Electron Microscopy

Particle suspensions were prepared at 10mg/mL in basic water (pH 9.0), and a 50 uL drop was put on an aluminum pin stub specimen mount (Ted Pella, Inc., Redding, CA) and allowed to air dry. The sample was sputter-coated with AuPd for 90 sec using a Sputter Coater 108 (Cressington Scientific Instruments, Watford, England). Images were acquired using a Nova NanoSEM 400 scanning electron microscope (Field Emission, Inc., Hillsboro, OR).

Endotoxin Levels

Particle suspensions were prepared at 1mg/mL in endotoxin free water and incubated overnight at 4 C. The amount of endotoxin was measured following the manufacturer's instructions using a Pierce LAL Chromogenic Endotoxin Quantification Kit (Thermo Fisher Scientific, Rockford, IL).

Cell Preparation

Macrophages (RAW 264.7; ATCC, Manassas, VA) were grown and maintained according to the manufacturer's guidelines. Media was made with fetal bovine serum (50 mL; Hyclone, Pittsburgh, PA), penicillin-streptomycin (5 mL; Fischer, Pittsburgh, PA), and Dulbecco's Modified Eagle's Medium (450 mL; Fischer, Pittsburgh, PA). Cells were maintained at 100% relative humidity, 37 °C, and 5% CO2. Macrophages were seeded at a concentration of 2×10^5 cells/mL in a 96-well plate. After 4 hours, the media in each well was replaced with treatment media containing blank Ace-IN and TMS-IN microparticles at concentrations ranging from 100 to 1000 µg/mL. Controls included three wells with only media, three wells with untreated cells, and three wells with 10 ug/ml LPS as a positive control. Cells were then incubated for 48 hours.

Cell Viability analysis.

After 48 hours of treatment, the MTT assay was performed to determine cell viability. Media was removed from wells and replaced with 150 uL of fresh media and 20 µL of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in medium (5 mg/mL) was added. The plate was incubated for 3 hours until purple formazan crystals formed. Then, the supernatants were removed and crystals were dissolved in isopropanol (200 µL). The plate was analyzed with a plate reader at an absorbance of 560 nm, and the background absorbance at 670 nm was subtracted. The net absorbance scaled by the net absorbance of the untreated cells (100%) corresponds to the cell viability.

Cytokine Analysis.

After 48 hours of treatment to the cells, supernatants from each well were removed and frozen at -80ºC. A Mouse TNF-α ELISA Ready-SET-Go!® kit (Affymetrix, Santa Clara, CA) was used to assess TNF-α Alpha levels in the samples. A 10 µL aliquot of each sample was pipetted into a 96

well plate for cytokine analysis. This and a Mouse IL-6 Ready-SET-Go!® kit (Affymetrix, Santa Clara, CA) ELISA were performed following the manufacturer's directions.

Nitrite Analysis

Nitric oxide production by the macrophages was measured using a Greiss Assay. After 48 hours, the supernatants from each well were removed and centrifuged (14,000 × *g*, 4 °C, 10 min), and 50 µL of the resulting supernatant was withdrawn and pipetted into a 96 well plate. Standard nitrite concentrations were prepared and the Greiss reagents (Promega, Madison, WI) were added following the manufacturer's directions. The absorbance was measured at 540 nm and compared with the standard curve to determine nitrite concentration.

Results and Discussion

Physical Characterization of Microparticles

Ace-IN and TMS-IN polymer was synthesized using the reaction schemes shown in Figure 1. Microparticles were synthesized from three reaction times for the Ace-IN polymer (20 min, 24 hour, 72 hour) and for the TMS-IN as seen in Figure 2. The microparticles were fairly polydisperse with diameters ranging from a few hundred nanometers to a couple microns. In order to ensure there was no contamination that could lead to false positives, particles were tested for endotoxin using a quantitative LAL Endotoxin test which resulted in a negligible amount of endotoxin being detected.

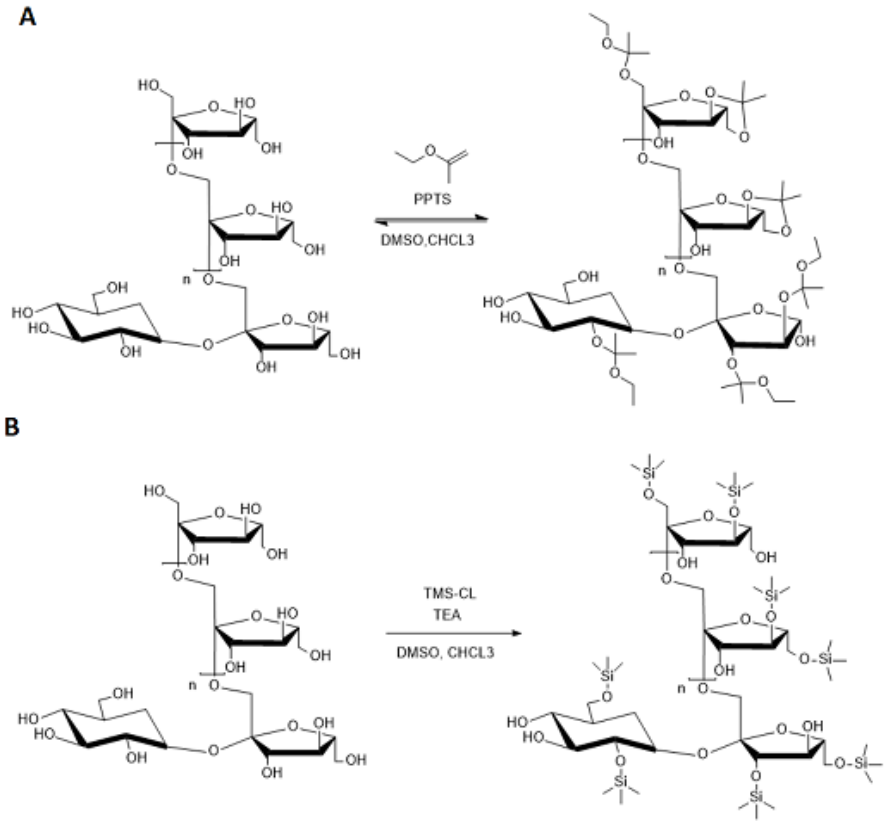

Figure 1: Reaction schemes used to synthesize: (A) Acetalated Inulin and (B) Trimethylsilyl Inulin

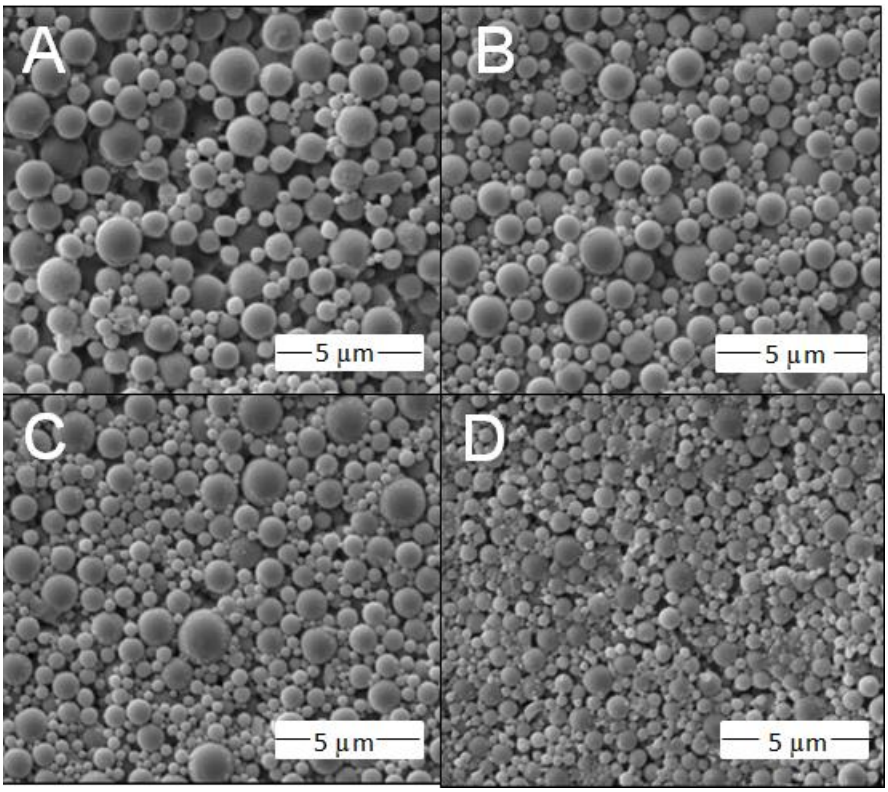

Figure 2: Scanning electron micrographs of blank microparticles fabricated by an oil/water emulsion: (A) Ace-IN 20min, (B) Ace-IN 24hr, (C) Ace-IN 72hr, and (D) TMS-IN. Scale bars = 5 µm.

Microparticle Degradation, Encapsulation Efficiency of Antigen, and Antigen Release

Reaction time has been shown to control the degradation rate of Ace-DEX particles.[29] It was expected that Ace-IN would have a similar degradation to acetalated dextran due to its acetal groups. The degradation of Ace-IN and TMS-IN microparticles synthesized using the emulsion method was analyzed in pH 5.0 and pH 7.4 buffers. The pH 5.0 buffer was used to model the physiological pH of phagosomal conditions of antigen presenting cells (e.g. macrophages) as well as sites of inflammation. Acid sensitivity is advantageous for a microparticle carrier since it allows for the payload to be localized to acidic sites (e.g. phagosomes) instead of physiological pH-neutral sites making delivery more efficient and reducing potential side effects intracellular.

Figure 3 shows the degradation of 20 minute Ace-IN particles, 24 hour Ace-IN particles, and TMS-IN particles.

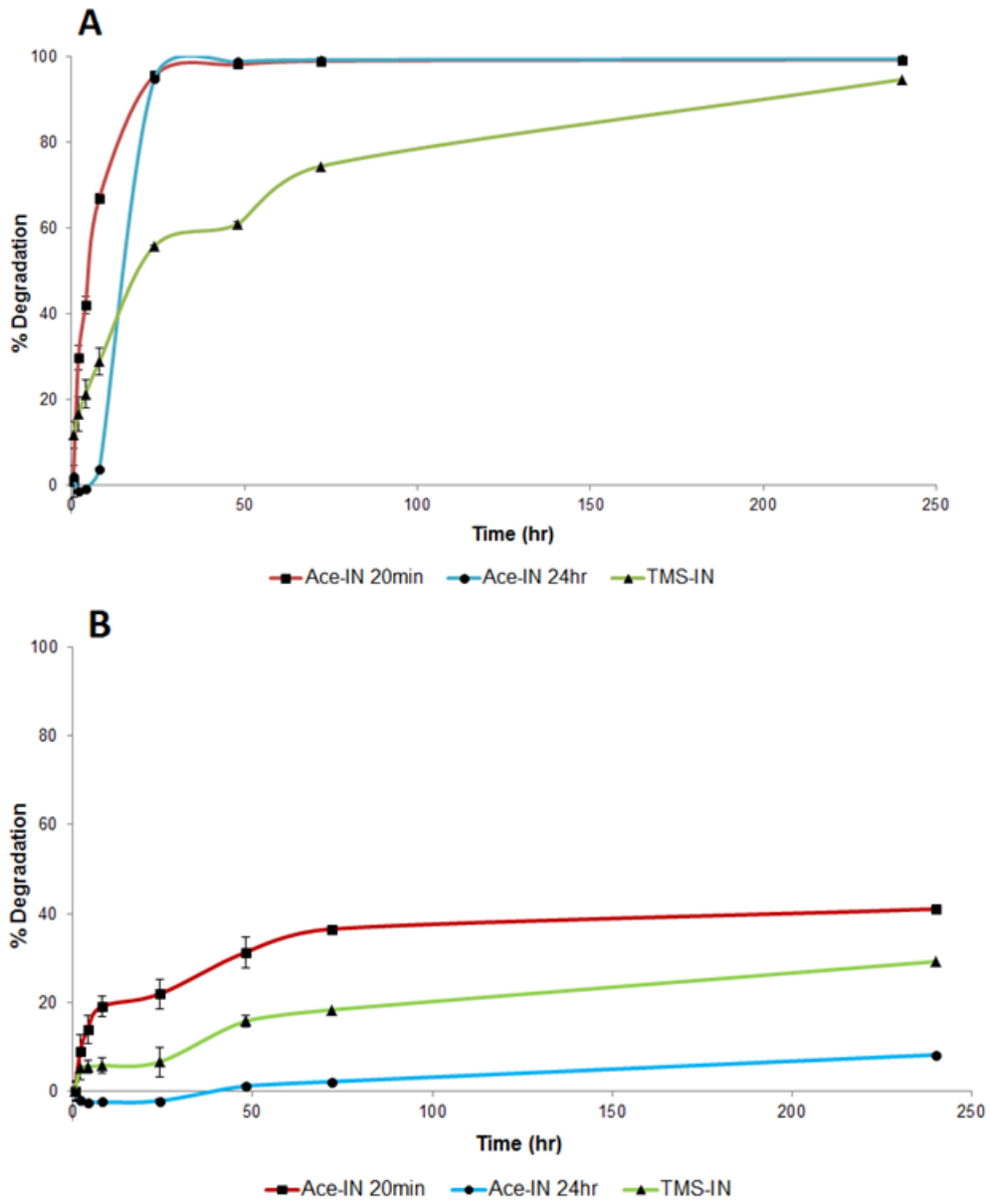

Figure 3: Degradation of blank inulin microparticles at 200 RPM, 37 ºC in (A) pH 5 Sodium Acetate buffer and (B) pH 7.4 Phosphate-Buffered Saline buffer performed at 200 rpm, 37ºC, *n*=3.

As expected, the lower reaction time Ace-IN degraded faster than the 24 hour Ace-IN particles, which shows degradation tunability based on the reaction time. Furthermore, TMS-IN particles

degraded slower than the Ace-IN particles, which implies an ability to further tune the degradation based on the chemical modification. All of the particles show a more triggered degradation in the pH 5.0 buffer as compared to being more stable at pH 7.4.

Double-emulsion microparticles loaded with OVA were prepared to investigate antigen release from the Ace-IN microparticles. The release is shown below in Figure 4. pH 5.5 was used instead of pH 5.0 to avoid OVA's isoelectric point and assure that it would remain in solution. A burst release was observed at the pH 5.5 and a slower release at pH 7.4 demonstrating the pH sensitivity and ability for the microparticles to release an antigen in a tunable fashion. This demonstrates the ability of particles to rapidly deliver antigen at a lower pH which has been shown to correspond with greater antigen presentation.[30]

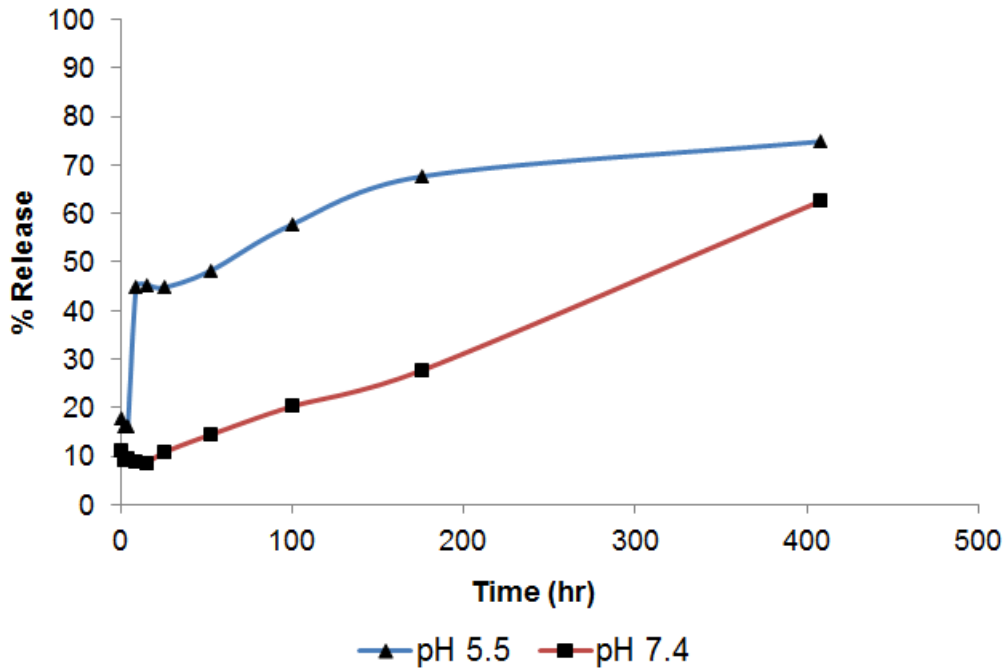

Figure 4: Release of ovalbumin (OVA) from Ace-IN: 72hr microparticles at pH 5.5, and pH 7.4 performed at 200 rpm, 37ºC, *n*=3.

After determining the degradation profile of the inulin microparticles, the ability of microparticles to encapsulate a model antigen (OVA) was tested. Issues arose with the encapsulation efficiency in TMS-IN, and therefore various polymer blends of Ace-DEX and TMS-IN were used to alleviate this problem. The encapsulation results can be seen in Table 1. With an increasing percentage of Ace-DEX, a non-immunostimulatory polymer, in the blend the encapsulation efficiency increased demonstrating tunability based on the amount of ovalbumin loading desired.

Table 1: Encapsulation Efficiency of OVA in TMS-IN/Ace-DEX blended microparticles (MPs).

Particle Type	Encapsulation Efficiency (%)
100% TMS-IN	4.28 ± 1.58
75% TMS-IN/25% Ace-DEX	14.02 ± 1.58
50% TMS-IN/50% Ace-DEX	27.04 ± 0.72
100% Ace-DEX	61.59 ± 0.86

Immune Characterization of Microparticles

Empty particles were tested for their cytotoxicity using the MTT assay and ability to induce an immune response using a tumor necrosis factor alpha (TNF-α) ELISA. TNF-α is a pro-inflammatory cytokine produced in response to infectious agents and has been shown to play an important role in immune and inflammatory activities.[31] Previously, it has been shown that inulin is immunostimulatory, so it was expected for the particles to result in cytokine release.[24]As seen in Figure 5, there was a significant TNF- α release with the Ace-IN particles. It can also be seen that there is a trend, with increasing reaction time resulting in a smaller TNF-α release. This demonstrates potential tunability for the degree of immunogenicity based on the reaction time of the polymer. One potential mechanism explaining this is a rapid degradation for lower reaction time acetalated inulin (as can be seen earlier in Figure 3) resulting in expedient delivery of inulin

to phagosomes for greater antigen presentation.[30]Additionally, there was a relatively high TNF-α released with treatment of TMS-IN particles. Viability for all the particles was above 80 percent for concentrations equal to or below 250 ug/ml, indicating that the particles are relatively not cytotoxic.

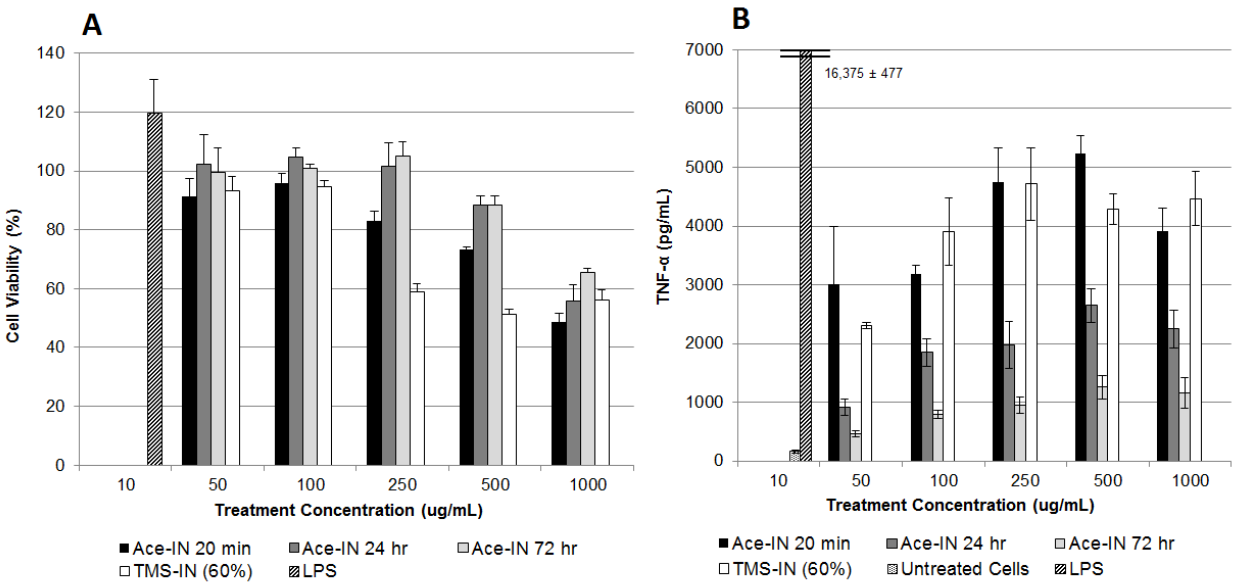

Figure 5: (A) Viability of RAW 264.7 macrophages determined by an MTT assay after 48 hours of treatment (B) Tumor necrosis factor alpha (TNF-α) levels produced by RAW 264.7 macrophages treated for 48 hours with blank microparticles. 10 µg/mL of lipopolysaccharide (LPS) was incubated for a reduced amount of time to act as a positive control. Data are displayed as mean ± standard deviation, *n*=3.

In Figure 6, the TNF-α production from free Inulin at the same concentrations as the particulate form of inulin is displayed. With free inulin, there was no TNF-α release seen for any of the concentrations. This indicates that especially when in microparticle form, inulin results in a Th1 immune response. This is consistent with the results that have been obtained previously by Kumar et al. using soluble inulin microparticles.[32]It can also be seen from the MTT results that free inulin, a degradation byproduct of the particles, is biocompatible.

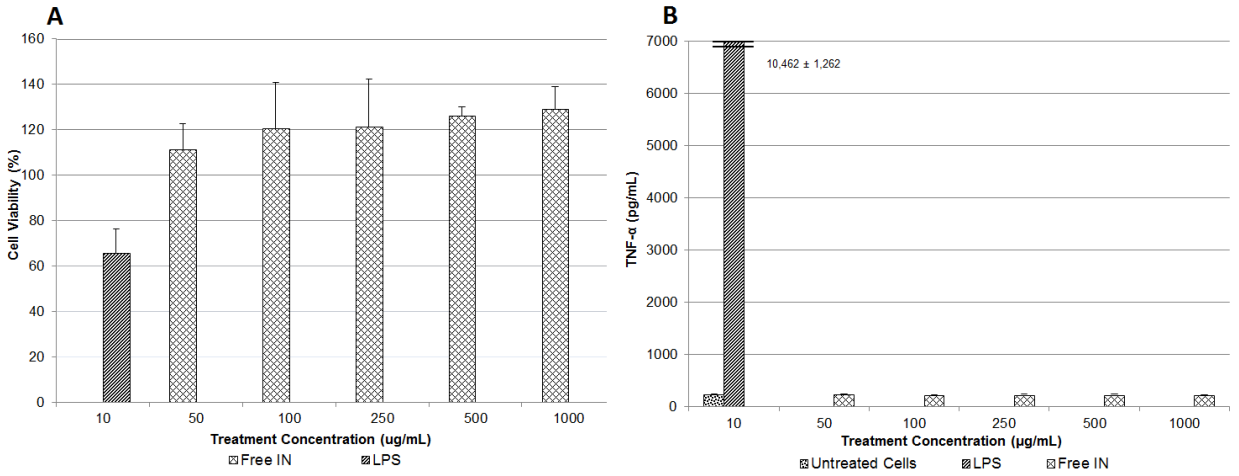

Figure 6: (A) Viability of RAW 264.7 macrophages determined by an MTT assay after 48 hours of treatment. Data are presented as relative to untreated cells and are displayed as mean ± standard deviation. (B) Tumor necrosis factor alpha (TNF-α) levels produced by RAW 264.7 macrophages treated for 48 hours with free inulin. 10 µg/mL of lipopolysaccharide (LPS) was incubated for a reduced amount of time to act as a positive control. Data are displayed as mean ± standard deviation, *n*=3.

Additional experiments assessing the ability for inulin to induce a Th1 response were performed including tests for IL-6 and nitric oxide, other inflammatory markers, but no response was detected as can be seen in Figures S-1, and S-2. Ace-IN blended with Ace-DEX microparticles were also tested for TNF-alpha emission as seen in Figure S-3.

In Vivo Experiments

After characterizing the physical and immune-modulating properties of inulin microparticles, microparticle degradation, and ability to encapsulate sample antigen, in vivo experiments were planned. *In vivo* experiments are ongoing and will characterize the humoral and cellular immune responses to the particles with anti-OVA antibody titers, flow cytometry and antigen recall. The encapsulation efficiency of the particles used in the in vivo experiment is displayed in Table 2.

Table 2: Encapsulation Efficiency of microparticles (MPs) used for *in vivo* experiment.

Particle Type	Encapsulation Efficiency (%)
24 hr Ace-IN	24.46 ± 0.66
50% TMS-In/50% Ace-DEX	27.04 ± 0.72
Ace-DEX	61.59 ± 0.86

The experimental groups chosen for the *in vivo* experiment are in Table 3. Delivery of OVA-loaded particles and free OVA in combination with blank particles were chosen with the appropriate controls.

Table 3: *In vivo* experimental groups

Group	
1	PBS
2	Blank Ace:IN:24hr MPs
3	Blank TMS:IN MPs
4	Free OVA
5	Free OVA + Alum
6	Free OVA + Ace:IN:24hr MPs
7	Free OVA + TMS:IN MPs
8	Ace:IN:24hr/OVA MPs
9	TMS:IN/OVA MPs
10	Ace-DEX:71k:50min/OVA MPs

The experimental plan for the *in vivo* experiment is displayed in Figure 7. Mice will be injected on Day 0 with a booster injection on Day 14 and will have blood samples taken every 2 weeks for 6 weeks total. Following euthanasia, the spleens will be excised and splenocytes recovered to determine the cell populations (e.g. CD8+) and ability of the splenocytes to recall the OVA antigen. Additionally, anti-OVA antibody titers will be analyzed to quantify the immune cell activation.

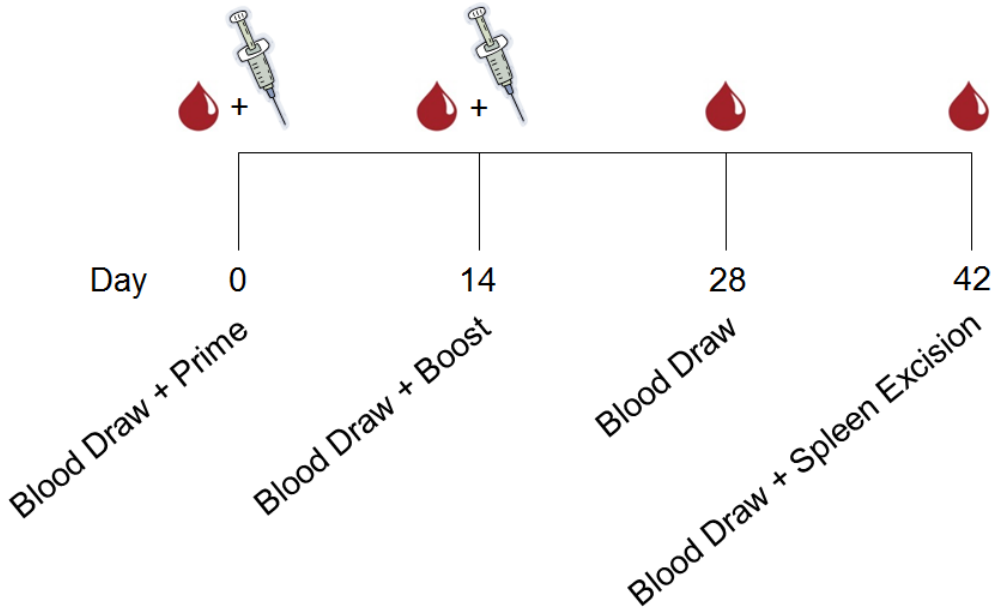

Figure 7: Schedule for *in vivo* experiment

Conclusions

It can be concluded that chemically-modified inulin has numerous properties that are optimal for a vaccine carrier. Both TMS-IN and Ace-IN show pH-sensitive characteristics beneficial for delivery of vaccines to phagocytic cells. Additionally, both types of microparticles are relatively biocompatible and immunostimulatory, causing a dose-dependent increase in TNF-alpha production. Due to the immunostimulatory effect of Inulin, these microparticles when formulated into a vaccine may not require a separate adjuvant drug. Tunability in degradation and immunogenicity has been demonstrated with Ace-IN particles based on the reaction time of the polymer synthesis. An increase in antigen loading can be achieved with TMS-IN by utilizing polymer blends containing Ace-DEX, another biocompatible polymer. Once results are obtained from the *in vivo* experiments, the effect of a chemically-modified inulin as a potential vaccine carrier can be further evaluated as part of a vaccine formulation for a specific disease.

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Supplementary Figures

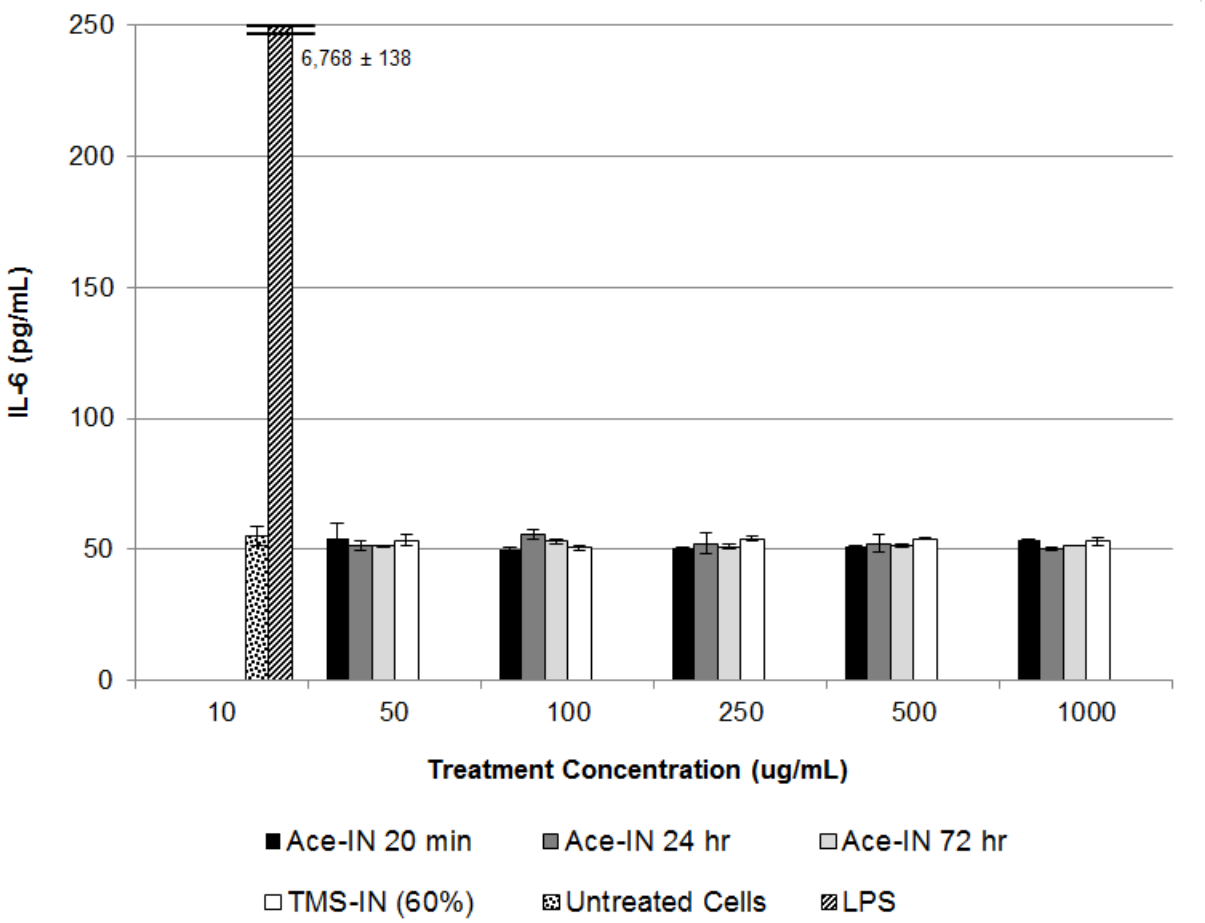

Figure S.1.: Interleukin-6 (IL-6) levels produced by RAW 264.7 macrophages treated for 48 hours with blank microparticles. 10 µg/mL of lipopolysaccharide (LPS) was incubated for a reduced amount of time to act as a positive control. Data are displayed as mean ± standard deviation.

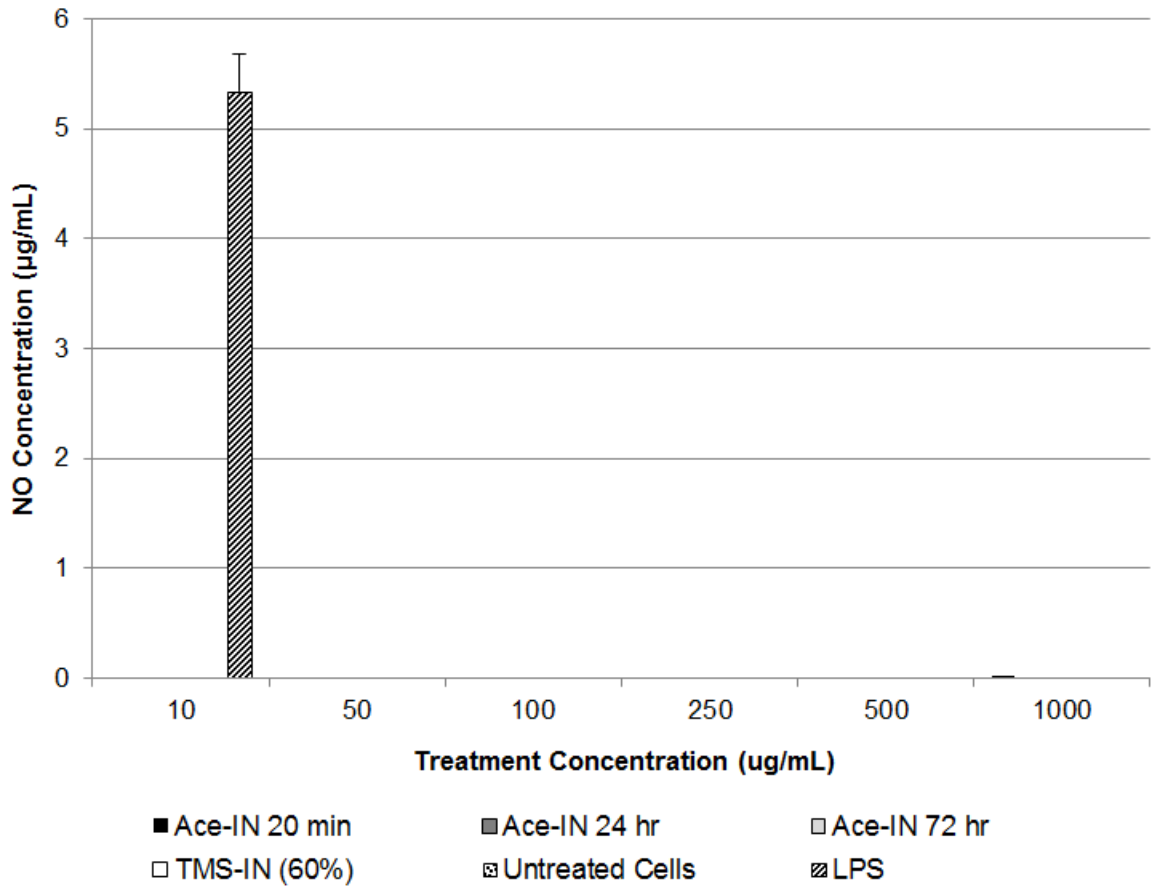

Figure S.2: Nitric Oxide (NO) levels produced by RAW 264.7 macrophages treated for 48 hours with blank microparticles. 10 µg/mL of lipopolysaccharide (LPS) was incubated for a reduced amount of time to act as a positive control.

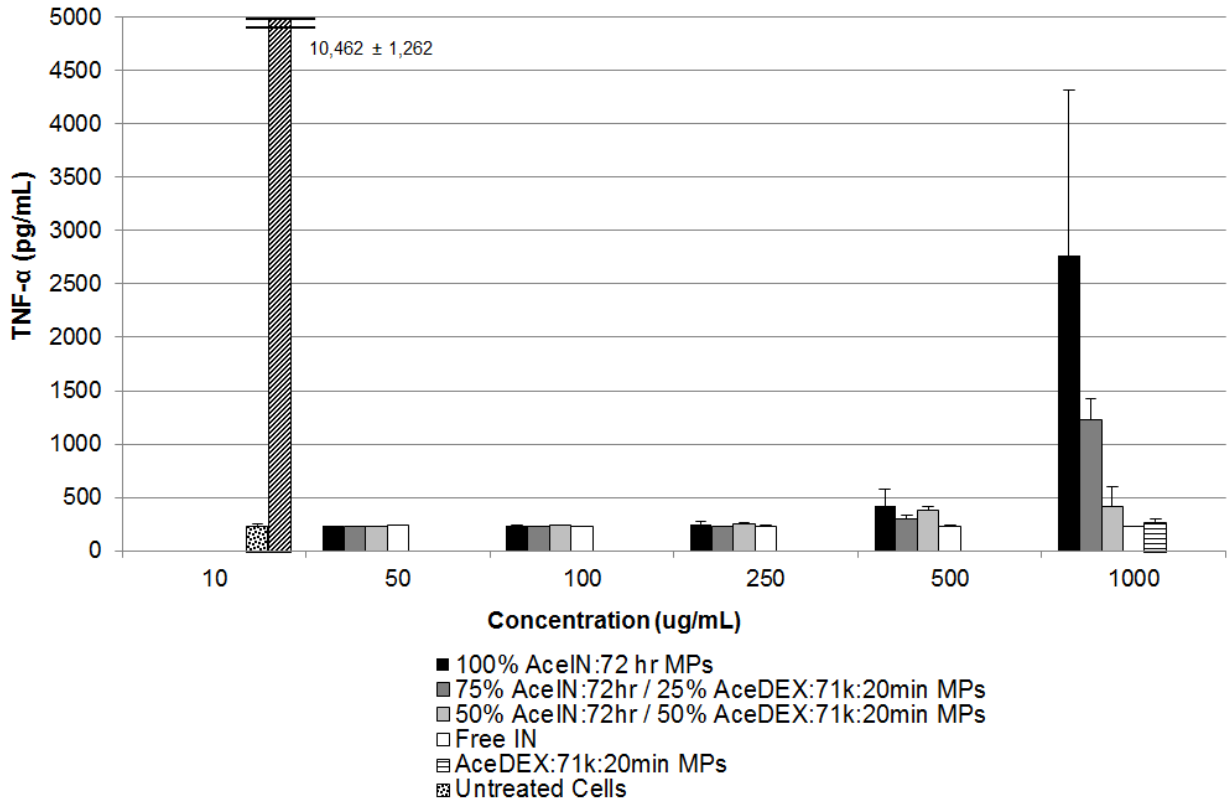

Figure S.3: Tumor necrosis factor alpha (TNF-α) levels produced by RAW 264.7 macrophages treated for 48 hours with blank microparticles blended with Ace-DEX:71k:20min. 10 µg/mL of lipopolysaccharide (LPS) was incubated for a reduced amount of time to act as a positive control.